Attorney's Docket No.: 08609-003004 / 920301 E

#6
12-6-00
PL

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Lawrence J. Wangh
Serial No. : 09/226,766
Filed : January 6, 1999
Title : IN VITRO ACTIVATION OF A NUCLEUS

Art Unit : 1632
Examiner : D. Crouch

Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF PROFESSOR WANGH

Lawrence J. Wangh, Ph. D. declares:

1. I am an Associate Professor at Brandeis University. At Brandeis, I have concentrated on reproductive biology in teaching and, particularly, my research for over twenty-five years.

2. I am the inventor of the method for improved cloning of the patent application identified above, which is owned by Brandeis University.

3. I have been shown the Official Letter from the Examiner dated May 23, 2000, and I have been asked to comment on the issue of whether or not my patent application contains teachings sufficient for persons skilled in the art to successfully clone whole animals without "undue experimentation". I am informed that the term "undue experimentation" means inventive contribution, not optimization, even if optimization requires a significant investment of time and effort. I have been asked to pay particular attention to the Examiner's statement on page 8 that the specification must supply the novel aspects of the invention in order to constitute adequate enablement.

4. To place the teaching of the patent application and the "novel aspects of the invention" in proper historical context, it is important to consider the state of cloning prior to the

CERTIFICATE OF MAILING BY FIRST CLASS MAIL

I hereby certify under 37 CFR §1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, D.C. 20231.

Nov 21- 2000
Date of Deposit

Francisco Robles
Signature

Francisco Robles
Typed or Printed Name of Person Signing Certificate

application. To assist in this, I will cite to a recent book by one of the leaders in the field, Marie Di Berardino. Chapter 3 of Dr. Di Berardino's book is attached as Exhibit 1.

5. Cloning has been known for many decades. Much of the history can be found in Di Berardino Chapter 3. In 1962 John Gurdon reported his successful cloning of whole animals: Gurdon, J.B., Adult Frogs derived from the Nuclei of Single Somatic Cells, Dev. Biol. 4:256-273 (1962), a copy of which is attached as Exhibit 2. A significant fact to keep in mind is that Gurdon's success was achieved with nuclei from a pre-metamorphic frog; that is, a tadpole. Gurdon could not reproduce these results using nuclei from a post-metamorphic frog.

6. From the historical account in Di Berardino Chapter 3, one can see that cloning techniques including nuclear transfer, enucleation of eggs and activation were developed and practiced successfully using embryonic cell nuclei as donor nuclei. One technique, serial cloning, referenced on page 93 is from the King and Briggs' 1956 paper whose citation appears at Di Berardino page 339. The pertinent fact is that repeatable success was obtained only with embryonic donor nuclei. See the graph at Di Berardino page 67, which illustrates how the ability to obtain larvae declined with advance in the stage of nuclei. (Pages 93, 339 and 67 are attached as Exhibit 3.)

7. It was recognized that there was some problem with the condition of nuclei. As indicated, Briggs and King utilized serial cloning. This cloning technique yielded greater uniformity, but it did not overcome the limitations observed with somatic cell nuclei. Di Berardino herself in 1986 reported transferring nuclei into first meiotic metaphase oocytes (surgically obtained oocytes at the first stage of maturation into eggs). When she combined this variant with serial transplantation into mature unfertilized eggs, she obtained tadpoles. See Di Berardino page 93. It must be noted that her donor cells for the second transplantation were triploid, that her initial donor cells were differentiated but quite young, and that there was uncertainly as to how many nuclei were injected in each case. Principally with mammals, serious attempts to overcome or avoid the perceived problem of nuclei conditioning were made by transplanting S-phase nuclei into already activated eggs. See Di Berardino Table 10.8 and pages 207 and 208, attached hereto as Exhibit 4. This technique increased the percentage of blastocysts, but not to lead to adult animals.

8. My patent application being examined teaches what was missing to permit embryonic cloning to be expanded to somatic cells. For definitional purposes I will refer to the language of claims 87 and 111.

Claim 87 states: the improvement comprising incubating said donor-cell nucleus in cytoplasms consisting of two types: first, in cytostatic factor- containing cytoplasm that is arrested in metaphase selected from the group consisting of meiotic metaphase II and mitotic metaphase; and subsequently in activated-egg cytoplasm.

Claim 111 states: prior to contacting the donor –cell nucleus with the cytoplasm that is arrested in metaphase, the cytoskeleton surrounding the donor-cell nucleus is structurally altered.

9. The patent application teaches how to perform these steps and how to apply them. For convenience, I will refer not to the typed version of the application but to the printed version in Wangh United States Patent No. 5,480,772, attached as Exhibit 5. At column 15, lines 45-54, for example, the application teaches that incubating non-dividing nuclei in unactivated egg cytoplasm is not a species-specific step; rather, it is universal. Further, mammalian and other sources for unactivated egg cytoplasm are presented at length at column 14, line 14 to column 15, line 29. Finally, the duration of incubation is functionally given at column 15, lines 32-35, events which are observable visually in mammalian nuclei. This step applies across species.

10. Unlike many of the earlier cloning publications, this application teaches incubation in unactivated egg cytoplasm at the molecular biology level. For example, the presence of cytostatic factor, or "CSF", is explained, as is mitosis promoting factor, "MPF", and histone H1 kinase activity. That teaching occurs generally at column 15, line 30 to column 17, line 30. From the teaching of the application persons skilled in the art would understand that the cytoplasm to use exists only in unactivated eggs and just-activated eggs, as the necessary chemistry declines rather promptly following activation.

11. The application also teaches preparation of non-dividing nuclei by two steps that may be carried out simultaneously or separately. These steps, as stated at column 9, lines 42 – 49, are (1) membrane permeabilzation and (2) separation or alteration (e.g., denature) of cytoskeletal proteins and nuclear matrix proteins. This aspect is also discussed at the biochemical level at column 9, line 9 to column 10 line 60. Use of lysolecithin, the protease

trypsin and heparin (a chelating agent), in mild concentrations to minimize damage to non-cytoskeletal proteins, histones and nucleic acid, is specifically stated as one way to perform this step. From the teaching persons skilled in the art would understand that in-vitro processing is required, what it is, and how to do it. As with the process step of claim 87, this step applies across species.

12. The nature of the starting nucleus is taught in numerous locations to be a non-dividing nucleus. See, for example, column 2, line 20; column 9, line 11-12 and column 23, line 28.

13. The nature of the recipient egg is discussed in column 23. The application instructs that standard transplantation techniques are to be used, with care taken that recipient eggs are handled so as to prevent activation prior to the transplantation procedure. Two references are cited: Gurdon, and King. Both reflect, that the standard procedure was to activate at or shortly after transplantation; that is, well prior to S-phase in the cell cycle. From the teachings discussed above, persons skilled in the art would understand that, if the recipient egg itself is being relied on to provide incubation in cytoplasm biochemically characterizable as "unactivated" (that is, the step of claim 87), transplantation without activation can be utilized. One skilled in the art would understand, therefore, where the recipient egg is in the cell cycle.

14. Based on the disclosures referenced above, and for the reasons given, the application contains teachings sufficient to enable persons skilled in the art to apply the novel aspects of the invention as set forth in the steps of claim 87 and claim 111. Evidence in the literature indicates that the method taught in this application for cloning starting with non-dividing cells has been applied with repeated success by workers in the art, particularly since the publication of our paper on this technology: Wangh et al., Efficient Reactivation of Xenopus Erythrocyte Nucleic in Xenopus Egg Extracts, J. Cell Sci. 108: 2187-2196 (1995) attached as Exhibit 6.

15. Wilmut et al. have successfully cloned sheep, Nature 385: 810-813 (Feb. 27, 1997); Time Magazine, March 10, 1997, pages 62-65, attached as Exhibits 7 and 8, respectively. Their procedure has been to render donor nuclei non-dividing by starving donor cells into quiescence (Go State) in tissue culture on a plate. Because Wilmut et al. do not discuss anything special about how they remove cells from the plate, one must conclude that they follow standard

procedure, which is the use of EDTA (a chelating agent) and trypsin. This is the performance of the procedure of claim 111. Wilmut et al. then fuse the donor nuclei with unactivated eggs, which would also alter the cytoskeleton, as the first observed event following fusion is dissolution of the nuclear envelope. Wilmut et al's. nuclear transfer is into unactivated eggs, followed subsequently by activation. Thus, the incubation of claim 87 is performed as well. A publication by Roslin Biomed, Exhibit 9, which I can date approximately by the facsimile date on my copy, June 17, 1999, is instructive. There, the use of the term "quiescence" is said to mean, "not actively dividing", which is what my application teaches. Further, incubating the donor nucleus in unactivate egg cytoplasm is described as key: "vital to give rise to an embryo capable of generating a live birth".

16. Yanagimachi et al. have successfully cloned mice. Nature 394: 369-374 (1998); Mol Reprod. Dev. 57 (1): 55-59 (Sept. 2000), attached as Exhibits 10 and 11, respectively. Referring to Fig. 5 of the Nature article, one sees that Yanagimachi et al. start with non-dividing cells for donor nuclei; cumulous cells are known to be in Go. Yanagimachi then perform the step of claim 111 by treating with enzyme (hyaluonidase) and breaking cells in a micropipette. Then Yanagimachi et al. perform the step of claim 87 by injecting the prepared nuclei into the MII-phase cytoplasm of unactivated eggs, incubating 1-6 hours, and then activating. In one of their publications, Nature Genetics 24: 108-109, Exhibit 12, Yanagimachi et al. present a graph verifying our teaching at the molecular biology level that CSF and MPF, for example, are critical to the step of claim 87. As indicated, it was known that the necessary chemistry declines following activation. Fig. 1 shows that the success rate is best, if activation is delayed 1-3 hours after nuclear transfer; that the success rate is lower but still rather good, if activation occurs with transfer; but that the success rate plummets, if activation precedes transfer by one hour.

17. Dr. Steven Stice and co-workers at Advanced Cell Technology have successfully cloned cows. Science 280: 1256-1258 (1998) (Exhibit 13); New York Times, national, page A14, (January 21, 1998) (Exhibit 14); United States Patent No. 5,945,577 (Exhibit 15); international applications WO 98/07841, 95/16770, 95/17500 (Exhibits 16-18, respectively). From the Science paper, one can see from Fig. 1 that fibroblasts are grown flat. Releasing them and "rounding them up" with trypsin and EDTA (a chelating agent), as discussed above in connection with Wilmut et al. and as presented in Example 1 of U.S. 5,945,597, is the

performance of the step of our claim 111. Referring to note 12 of the Science paper, one sees that prepared nuclei were fused with unactivated eggs, that is, in MII-phase cytoplasm, followed after 2-4 hours by activation. The donor nuclei are non-dividing as that term is used and explained in my patent application, which teaches treatments to take a nucleus that is not making DNA ("DNA replication") and by the steps of claims 111 and 87 cause that nucleus to synthesize DNA efficiently.

18. Several groups have successfully cloned pigs. A paper in Nature 407: 86-90 (2000) (Exhibit 19) reported that quiescent cultured cells were lifted off the culture plate (as explained above, the step of claim 111) fused with MII-phase eggs, and subsequently activating. Koo et al., Biol. Reprod. 63(4): 986-992 (2000) (Exhibit 20) similarly reported transplantation into unactivated eggs (MII-phase cytoplasm) and subsequently activating. Kuhholzer et al., Mol. Reprod. Dev. 56 (2): 145-148 (2000) (Exhibit 21) also similarly reported nuclear transfer into unactivated eggs with subsequent activation.

19. Ogura et al. have successfully cloned mice from Sertoli cells, which are non-dividing or "quiescent". Biol. Reprod. 62(6): 1579-1584 (2000) (Exhibit 22). The required tissue disruption is understood to perform the step of claim 111. Then the prepared nuclei are transferred to MII-phase oocytes and subsequently activated.

20. Shiga et al. have successfully cloned cows. Theriogenology 52(3): 527-535 (1999) (Exhibit 23). Donor nuclei from quiescent cells were transferred both into unactivated eggs and into activated eggs. The former, which entails incubation in MII-phase cytoplasm according to claim 87, worked; the latter did not.

21. Wells et al. have successfully cloned cows from non-dividing cell nuclei by transferring nuclei from quiescent cells into unactivated eggs followed by prolonged exposure to MII-phase cytoplasm. Wells et al. Reprod. Fertil. Dev. 10(4): 369-378 (1998) and Biol. Reprod. 60(4): 996-1005 (1999) (Exhibit 24 and 25, respectively). Zakharchenko et al. reported successful cloning of cows with fetal germ cells by fusing them oocytes, incubating for 2-4 hours, and then activating Mol. Reprod. Dev. 52 (4): 421-426 (1999) (Exhibit 26).

22. In a review paper by Kikyo and Wolffe of NIH, Reprogramming Nuclei: Insights from Cloning, Nuclear Transfer and Heterokaryons, J. Cell Sci. 113: 11-20 (2000) (Exhibit 27), the authors recognize (page 15) that the "unifying aspect of the reprogramming of somatic nuclei

following their transfer into the egg [the step of claim 87] is that the biochemical changes establishing constraints on genetic potential are reversed. Further, the authors cite our 1995 paper as documenting " the reacquisition of replication competence in Xenopus erythrocyte nuclei incubated in egg extracts."(id)

23. Since filing of my patent application, my laboratory has pursued, albeit with severe funding constraints, work with the Xenopus system toward the goal of a true biochemical understanding of the events of nuclear transplantation and cloning. While Xenopus presents practical problems, largely mechanical in nature, in practically optimizing techniques, certain rigorous constraints of the system can actually be helpful in reaching a true biochemical understanding. For example, the Xenopus egg is large and opaque, having a large amount of yolk. With a single optic system, it is not possible to view the egg nucleus, the donor-cell nucleus and the egg all at once. Because of the yolk, it is difficult to site a nucleus into the relatively tiny volume of egg cytoplasm. However, despite the lack of commercial significance of Xenopus, the rapidity of the Xenopus cell cycle may actually assist in optimizing aspects of the procedure, because small vaiations show up in this system. The first cell cycle in Xenopus is only 90 minutes, compared to several hours in mammalian systems, and the next eleven cycles are only 30 minutes, roughly half of which are S-phase. Moreover, cell cycle speed in amphibians is functionally independent of the nucleus. This presents a system suited to identify small effects on speed of DNA replication. We have studied, for example, the role of chelating agents in the step of claim 111, as well as the role of the level of H1 kinase activity. We are also studying the effects of performing the step of claim 87 partly in vitro and partly in vivo, as that provides a way to obtain enough material from homogeneous populations of cell nuclei for step-by-step biochemical analysis. Also, in our studies we have utilized erythrocytes as donor cells, for the reason that erythrocyte nuclei are seen to present the most stringent case for optimizing techniques, given their very nature.

I hereby declare that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are

punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: Nov. 21, 2000

Lawrence J. Wangh, Ph.D.

30053242.doc